UNIVERSITY OF CALIFORNIA, SAN DIEGO

# 14-3-3zeta mediates the recruitment of ARHGEF2 onto ROR1 to facilitate Wnt5a-induced CLL proliferation/migration

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by

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The Thesis of Lijia Zhang is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

In dedication to my mother, Jianrong Hao, and father, Dianying Zhang, for their support for my pursuit of science and medicine both spiritually and emotionally in the past, present, and future years. I am forever grateful.

In recognition of the members of Kipps lab for their assistance in this project—Dr. Thomas Kipps for mentoring me and giving me the opportunity to explore and study cancer biology; Dr. Jian Yu for his irreplaceable guidance and direction and for putting up with my forgetfulness and mistakes; Ling Zhang for her assistance and willingness to help and answer my many questions; and to rest of the many lab members not mentioned, your help was both invaluable and humbling to me.

In recognition of Dr. Donger Zhang and Dr. Lifan Lu for serving on my thesis committee. Thank you for your time and cooperation through the scheduling and bureaucratic business.

EPIGRAPH

We must never feel disarmed: nature is immense and complex, but it is not impermeable to intelligence; we must circle around it, pierce and probe it, looking for the opening or making it.

-Primo Levi, The Periodic

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ABSTRACT OF THE THESIS

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Chronic lymphocytic leukemia (CLL) is one of the most common leukemia that derives from the mutations of B lymphocyte. Previous research has shown that the receptor tyrosine kinase-like orphan receptor 1(ROR1), the receptor expressed specifically by the CLL, is induced to recruit guanine nucleotide exchange factors 2 (ARHGEF2) once activated by Wnt5a, the ligand that bind and activates ROR1 to promote the proliferation and migration of CLL. However, the mechanism for recruiting ARHGEF2 remain unclear. In this study, we found that 14-3-3ζ, one isomer of the 14-3-3 scaffold protein family, helps to recruit guanine nucleotide exchange factor 2 (ARHGEF2), on to

the ROR1 receptor in CLL. Here, we reveal that 14-3-3ζ interacts with both ROR1 and ARHGEF2 separately in CLL. Upon Wnt5a stimulation, 14-3-3ζ is anchored onto ROR1 via its S857 motif. Also, 14-3-3ζ is required for the Wnt5a-induced recruitment of ARHGEF2 onto ROR1. Lastly, 14-3-3ζ is necessary for the RhoA/Rac1 activation by ARHGEF2 and proliferation/migration induced by RhoA/Rac1 activation. Collectively, 14-3-3ζ mediates the recruitment of ARHGEF2 onto ROR1, which facilitates the Wnt5a-induced CLL proliferation/migration.

# INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in western world. The characteristic features of patient with CLL include lymphadenopathy (enlarged lymph nodes), fatigue, and weight loss. The majority of the affected patients consists of elders above 60 years of age, with more male than female patient being affected (Rozman and Montserrat, 1995). The medium survival length of CLL patients is approximately nine years. The variation of their life span, on the other hand, is relatively large: some patients with CLL have a normal life span, while other patients live no more than five years after diagnosis (Rozman and Montserrat, 1995).

CLL cell has extremely strong proliferate ability because it survives longer and undergoes apoptosis at the later life stage (Rozman and Montserrat, 1995). Thus, an absolute lymphocytosis (an increase in the number or proportion of lymphocytes in the blood) in patient is a distinct hallmark for CLL diagnosis. Furthermore, the lymphocytes have a typical morphology of small and mature shape in patient with CLL. Another feature of the CLL disease is that the lymphocyte infiltrate into the bone marrow, which causes anemia and thrombocytopenia in CLL patient (Cheson et al., 1988). Normally, the B cells present in the mantle zone of lymph nodes and blood. However, the neoplastic B lymphocytes (CLL cell) accumulate in the lymph nodes, spleen and liver because of the lymphocytosis. As a result, CLL patients develop lymphadenopathy, splenomegaly, and hepatomegaly (enlargement of lymph node, spleen and liver) (Rozman and Montserrat, 1995

The CLL cell derives from B-1a cell, a subpopulation of B lymphocytes that expresse the antigen CD5 (Gale el al., 1994). Normally, CD5 is expressed by T lymphocytes but not by B lymphocytes. However, B-1a cell is a special type of B lymphocyte that is CD5 positive. Multiple mutations of the B-1a cells have been found to contribute to the development of this type of leukemia. The overexpression of anti-apoptotic protein such as B-cell lymphoma 2 (Bcl2) and c-Myc in B-1a cells inhibit apoptosis of B-1a cell.(Hanada et al., 1993). Also, the mutation/deletion of the pro-apoptotic protein including tumor suppressor protein p53 (p53) and retinoblastoma protein (RB1) prolong the survival of B-1a cells (El Rouby et al., 1993; Matolcsy et al., 1994). Collaboratively, these mutations trigger the transformation of B-1a cells into CLL.

Immunophenotype, the trait that refers to the types of protein expressed on the cells surface, plays an important role in characterizing the identity of CLL cells. As the neoplastic lymphoma that derives from the B-1a cells, CLL expresses not only the antigens of regular B cell including CD19, CD20, CD22, and CD23 but also the antigen CD5 (Matutes et al., 1994). In this way, the CD5 expression become one of the most representative criteria to distinguish CLL from regular B cell. Another difference of the immunophenotype between B Lymphocyte and CLL is that they express different level of immunoglobulin, the antibody used by the immune system to identify and neutralize pathogens from the outer environment. While mature B cell present a certain amount of surface immunoglobulins such as IgM and IgD for secretion, CLL resemble the immature type of B cell which express lower level immunoglobulin (Matutes et al., 1994).

Ror1, the receptor tyrosine kinase-like orphan receptor 1, was identified as another protein that is expressed specifically on the surface of CLL but not on those of normal adult tissues or blood mononuclear cells (Fukuda et al., 2008). Ror1 protein is a transmembrane receptor that belongs to the receptor tyrosine kinase (Trk) family. Ror1 together with its ortholog Ror2 comprise the ROR receptor family (Masiakowski and Carroll R, 1992). As an evolutionary conserved cell surface protein, Ror1 is normally expressed in neural crest cells and mesenchymal cells during the embryogenesis of an organism (Yoda et al., 2003; Matsuda et al., 2001). In the early stage of fetal development, its expression contributes to the organogenesis of circulatory, skeletal, and nerve system (Al-Shawi et al., 2001). Other than its expression in CLL, Ror1 was also found to be expressed by a variety of other cancer including breast cancer and ovarian cancer (Zhang, et al., 2012; Zhang, et al., 2012; Zhang, et al., 2014).

Both Ror1 and Ror2 contain an Immunoglobulin-like domain, a Frizzled domain that rich in cysteine, and a Kringle domain within the extracellular region (Masiakowski and Carroll R, 1992). In the cytoplasmic compartment, they also have a tyrosine kinase domain and proline-rich domain(PRD) (Masiakowski and Carroll R, 1992). Ror1 participates in Wnt signaling pathway because the extracellular cysteine rich Frizzled domain acts as binding site for Wnt ligands (Angers and Moon, 2009). The binding of Ror1 by Wnt5a (one type of Wnt ligand) induces the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the protein complex that is associated with enhanced CLL survival (Fukuda et al., 2008). On the other hand, the Ror1 activation by wnt5 also triggers the phosphorylation of Protein Kinase B (AKT) and cAMP-response-

element-binding protein (CREB), which is correlated with enhanced tumor growth (Zhang, et al., 2012).

Similarly, Ror2 also interacts with the members of Wnt family ligands due to its extracellular cysteine rich Frizzled domain. (Oishi et al., 2003). The binding of Wnt ligand with Ror1 and Ror2 induces the oligomerization of these two receptors via their intracellular Kringle domains. This complex formation in turn recruit guanine nucleotide exchange factors (GEFs). As a result, GEFs activates Ras-related C3 botulinum toxin substrate 1(Rac1) and Ras homolog gene family member A (RhoA) by exchanging their previously associated GDPs with GTPs, which ultimately leads to enhanced proliferation and chemokine-induced migration of CLL (Yu et al., 2015). However, the mechanism that is used to recruit GEFs onto the Wnt5a-induced ROR1-ROR2 heterooligomer remains to be elucidated.

It is possible that an unknown protein with adapting property might help to mediate this interaction. As the protein that is widely used to facilitate the binding between two or more ligands (Hermeking, 2003), 14-3-3 is a good candidate that serve this function. 14-3-3 protein comprises a large family of small and acidic polypeptides of 28–33 kDa that are conserved in all eukaryotic species (Aitken et al., 1992). Seven isomers in 14-3-3 protein family including “beta” “epsilon” “gamma” “eta” “tau” “zeta” “sigma” have been identified (Neal and Yu, 2010). Previous studies show that isomers of the 14-3-3 protein family bind and regulate the activity of their corresponding ligands to mediates the cellular signaling pathways that are important for cancer progression (Hermeking, 2003). For instance, 14-3-3ζ triggers the transformation of Ductal Carcinoma

in Situ to Invasive Breast Cancer by interacting with TβRI to activate the TGFβ/Smads pathway (Lu et al., 2009). More importantly, some isomers of the 14-3-3 protein family such as 14-3-3β has been show to mediate the complex formation between multiple proteins such as Bcr and Raf (Braselmann and McCormick, 1995). In this study, we propose that the protein 14-3-3ζ mediates the recruitment of Rho guanine nucleotide exchange factor 2 (ARHGEF2), one specific subtype of GEFs, on to the ROR1 receptor in CLL.

To test our hypothesis, we begin with investigating the role 14-3-3ζ acts in contributing to the disease progression of CLL by correlating its expression level with the CLL aggressiveness. After confirming the physical binding between ROR1 and 14-3-3ζ, we compare the amino acid sequence of ROR1 with those of known 14-3-3ζ binding ligands to identify the potential 14-3-3ζ binding site on ROR1 receptor. To further investigate the interaction between ROR1 and 14-3-3ζ though the suspected binding site, we generate the MEC1 cell line that expresses the ROR1 receptor with the mutated 14-3-3ζ binding site. If the interaction between ROR1 and 14-3-3ζ is confirmed, we will exam the Wnt5a induced anchoring of 14-3-3ζ onto ROR1 by activating/blocking ROR1 receptor using Wnt5a/anti-ROR1 antibody and measuring the localization of ROR1 with 14-3-3ζ. Finally, we measure the interaction between ROR1 and ARHGEF2 in responds to the knock-out of 14-3-3ζ, which tells us whether 14-3-3ζ is used to adapt ARHGEF2 on to ROR1 receptor. Our result shows that 14-3-3ζ, the protein that contribute to the CLL development, is anchored on to ROR1 receptor through the S857 motif once activated by Wnt5a, which in turn recruit ARHGEF2 for further downstream interactions in CLL.

# METHODS

## CLL specimens

To obtain the fresh human CLL cells, the blood samples were collected from CLL patient at the Moores Cancer Center of UCSD. The Ficoll-Paque PLUS (GE Healthcare Life Sciences) was used to isolate the peripheral blood mononuclear cells (PBMCs) through density centrifugation. Based on the analysis of flow cytometry result, the samples of PBMCs with more than 95% of CD19+CD5+ CLL cells were used directly as the CLL specimens for this study without further purification. To store the samples, the isolated CLL cells were resuspended in freezing medium(FM) made with 90% of FBS (Omega Scientific) and 10% of DMSO (Sigma-Aldrich)

## Immunoprecipitation analysis

2^107 of freshly isolated CLL or MEC-ROR1 with or without the S857 point mutation were lysed in a prepared buffer made with 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, and Protease Inhibitor Cocktail (Roche Applied Sciences). The lysates were then pelleted by centrifugation at 16,000 g for 15 minutes, and the supernatants were taken and incubated with the protein G/A agarose beads that is linked to the anti-ARHGEF2 antibody (Abcam), anti-14-3-3ζ antibody (R&D), and anti-ROR1 antibody generated in our lab overnight at 4°C.

## Immunoblot analysis

Equal amount of protein samples prepared from the immune precipitate assay were added with 5× loading buffer (4%SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue,a nd 0.125M Tris-HCl) and heated for 8 minutes at 95°C. The boiled samples were loaded onto the SDS-PAGE gel and the protein contents were fractionated by the voltage that was applied across the

electrodes. After being transferred to polyvinylidene difluoride membranes and blocked using the PBS with 5% milk, the Size-separated proteins were probed with anti-ARHGEF2 antibody (Abcam), anti-14-3-3ζ antibody (R&D), and the mAb specified for ROR1 that was generated in lab. With three times of TBST wash, the probed proteins were further incubated with the secondary anti-mice/rabbit antibody conjugated with horseradish peroxide (Cell Signaling Technology). Lastly, the Femto/ECL blotting substrate (Thermos Scientific) were applied for detection of chemiluminescence through autoradiography.

**MS analysis**

Initially, the anti-ROR1 immune precipitate prepared from the freshly isolated CLL and MEC-ROR1 were washed by 1 ml of HEPES buffer 3 times (Thermo Fisher) and suspended in 100 µl HEPES buffer (50 mM; pH 7.2). Cystines within the beads-bound protein were reduced using 1 mM Tris (2-carboxyethyl) phosphine (Fisher) at 95°C for 5 minutes and then alkylated using 2.5 mM iodoacetamide (Fisher) at 37 °C in darkness for 15 minutes. Later, proteins were digested with 0.5 µg trypsin (Roche) with the ratio of 50:1 overnight. To isolated the digested peptide, supernatant of the trypsin treated

immune precipitate was collected and passed through a 0.22-μM filter via centrifugation. Then, digested peptides were separated by online 2D-nanoLC and detected by LTQ linear ion trap mass spectrometers. Each sample took 22.5 hours to analyze and about 200,000 MS/MS spectra were collected for each run. Raw data were extracted and searched using Spectrum Mill (Agilent, v3.03) database search software against the NCBI refseq database limited to human taxonomy (version 44).

**Nucleofection of siRNA and plasmid**

5 × 106 of freshly isolated CLL or MEC1 were suspended in 100ul of Nucleofector solution that contains 5ug of either Control/14-3-ζ siRNA (Life Technologies) or reconstructed plasmid for CRISPER. Afterward, the program U-015 of Nucleofector II device(Lonza) was used to electrically penetrate the cell membrane and to allow the transfection of siRNA to occur. After being transferred into 12-well plate in complete RPMI-1640 medium, the CLL cells were incubated in 37°C for 48 hours. To confirm the successful knock-down of 14-3-3ζ in CLL, the immune blot assay was used to access the 14-3-3ζ expression of the transfected CLL.

**Confocal microscopy imaging**

After receiving the combined treatment of Wnt5a and UC-961, the CLL cells transfected with Ctrl-siRNA or 14-3-3ζ siRNAs were stained with Alexa Fluor-647 conjugated anti-ROR1 mAb in PBS with 3% BSA. After 20 minutes of incubation at 4°C, the cells were washed twice with PBS and fixed with 4% paraformaldehyde with the same condition of the ROR1 staining. Following two times of PBS wash, the HBSS with 0.1% of

Saponin was used to permeabilize the stained cell for 10 minutes at 4°C. The CLL cells were then washed by two more time with PBS before blocked with 5% BSA in PBS at 4°C for 30 minutes. Later, the CLL cells were stained with either anti-14-3-3ζ antibody (R&D) or anti-ARHGEF2 antibody (Abcam) overnight at 4°C. On the second day, the CLLs were washed twice and stained with goat anti-rabbit secondary antibody (Alexa Fluor 594) for 60 minutes at 20°C. With two more times of PBS wash, the stained CLL cells were resuspend in 15ul of mounting buffer and attached to slides. Confocal images were collected on a Nikon A1R confocal microscope using a ×100 objective with an NA of 1.4. The laser lines for the different fluorophores were as follows: 405 nm for DAPI, 561 nm for Alexa Fluor 594, and 647 nm for Alexa Fluor 647. The percentage of the area that had colocalized signals was analyzed by ImageJ colocalization plugin. Colocalized regions were measured in pixels and related to the total cell area.

**GEF nucleotide exchange activity assay**

Cultured CLL cell transfected with either control siRNA or anti-14-3-3 siRNA was initially treated with Wnt5a(200ng/ml) for two hours in 37°C. To generate the anti-ARHGEF2 immune precipitate, the treated CLL cells were lysed and probed with bead that targets ARHGEF2 specifically. After the exchange buffer with His-tagged RhoA/Rac1 and -methylanthraniloyl-GTP (mant-GTP) was plated in 96-well plate, the Tecan Spectrofluor plus fluorimeter (λex = 360 nm, λem = 460 nm) was used to measure the exchange of GEF based on the enhancement of mant-GTP fluorescent intensity (ex: 360 nm, em: 440 nm). The plate was read by the fluorimeter every 30 second at 20°C for 44 minutes totally, and the prepared anti-ARHGEF2 immune were added into the plate 120 seconds after the

reading was initiated. Later, the exchange curve was generated by exporting raw data to Microsoft Excel and analyzing the data using GraphPad Prism 6.0.

**Heat shock transformation**

The ligated plasmid (10ng in 5ul) was mixed with 5-alpha Competent E. coli with the volume ratio of 1:10, and the mixture was incubated at 4°C for 30min followed by 30 seconds of heat shock in 42°C. After being diluted for 10 times with the addition of SOC, the transformed E.coli was incubated for 1 hour at 37°C followed by the overnight culture on LB agar plate. To amplify the ligated vector in the E. coli, the single clone from the plate was pick to be cultured in 500ml LB media overnight at 37°C.

**CRISPER assay**

We artificially construction the single strand oligo DNAs that resemble the second exon of 14-3-3ζ gene with the following sequences:

up: AGCCCGTAGGTCATCTTGGA

down: TCCAAGATGACCTACGGGCT

To generate the double strand DNA that targets 14-3-3ζ gene, one volume of each of the 14-3-3ζ targeting forward and reverse oligo DNAs were annealed together by incubating with T4 ligation buffer, T4 PNK, and ddH20 with the ratio of 2:1:13 in 37°C for 30 mins and 95°C for 5 mins. Later, the oligo duplex diluted by 250 times was ligated with the same amount of Cas9/GFP expressing Px330 plasmid with the help of Bbs1 restriction

enzyme and T4 DNA ligase at ratio of 2:1 in 5 mins of 37°C and 25°C separately for 6 cycles totally.

The vector ligated with the oligo duplex was transformed into E-coli though heat shock assay for the selection of amplified single clonal ligated plasmid. After being purified from the E. coli using the Qiagen Mega Prep Kit, the plasmid was transfected into the MEC1/MEC-ROR1 cell using the Nucleofector Kit. Then, the successfully transfected MEC1/MEC-ROR1 cell was sorted out based on the fluorescence emitted by GFP expressed by the Px330 vector. Later, the sorted MEC1/MEC-ROR1 cell was plated onto the semisolid medium for the selection of the MEC1/MEC-ROR1 with single mutation of 14-3-3 gene induced by the oligo DNA/Cas9 CRISPER system.

**RhoA and Rac1 activation assay**

According to the manufactory's instruction of Cytoskeleton, 2^107 of CLL transfected with Ctrl-siRNA or 14-3-3ζ-siRNA and MEC1/MEC-ROR1 with or without the knock-out of 14-3-3ζ were digested by the lysis buffer and centrifuged with 10000g for removing of cell debris. Part of the supernatant was taken and probed by the Rhotekin-RBD or PAK-PBD beads overnight at 4°C for pulling down of the GTP-bound Rac1 and RhoA. Later, both the protein samples with and without the bead-probing were fractionated on electrophoresis gel and incubated with the anti-RhoA antibody and anti-Rac1 antibody (Cytoskeleton) for detecting the activated and total RhoA/Rac1 in MEC1 and CLL. To quantify activated versus total RhoA and Rac1, the integrated optical density (IOD) of bands from the immunoblot result was analyzed by image J software. The activation level

of RhoA and Rac1 was calculated as the ratio of the band density for activated versus total RhoA/Rac1 normalized to that of the WT MEC1 and none-treated CLL transfected with Ctrl-siRNA.

**cell proliferation assay**

The MEC1/MEC-ROR1 with or without the knock-out of 14-3-3ζ were plated in 96-well plate with the concentration of 2^104 cell/well in complete RPMI-1640 medium. After being incubated in 37°C for a 3-day period, the MEC/MEC-ROR1 cell in each of the vial was applied with 10ul of CCK-8 solution followed by 1.5 hour of incubation in 37°C. Later, the OD value of the cultured cell was measured by the microplate reader with the absorbance at 450nm. The calibration curve used to convert the OD value to the cell count per well is prepared by matching the cell with known concentration ranging from 1^105/ml to 16^105/ml to their corresponding OD values using the CCK kit above.

**Chemotaxis induced migration assay**

5^105 of MEC1/MEC-ROR1 in RPMI-1640 medium were added into 8.0um polycarbonate membrane containing inserts. Then, each of the inserts was added on to top of the 24-well plate with each cell previously loaded with 100ng/ml of CCL21 in RPMI. Later, the plate with the 6.5mm insert was incubated in 37°C for 24 hours for the CCL21-induced migration of cell across the polycarbonate membrane. To determine the amount of MEC1/MEC-ROR1 in each cell of the 24-well plate after incubation, the cell concentration obtained from flow cytometry was multiplied by the volume of medium in

each well. Finally, the percentage of migrated cell was calculated as the amount of cell in 24-well plate divided by the total amount of cell applied initially into the insert.

**In vivo engraftment assay**

A total number of 5^106 MEC1 or MEC-ROR1 cell with or without the knock-out of 14-3-3ζ was injected into Rag2−/−γc−/− via i.v infusion (n=6). On days 16, all the mice were sacrificed and the spleens were taken for weight and picture. Later, the spleens were made into single cell suspension of splenocytes in HBSS. After the total number of cell in the spleen of each mouse was counted under the microscope, the splenocytes were resuspended in PBS (2% BSA) and stained with Alexa Fluor-647 conjugated anti-ROR1 mAb and PE conjugated anti-CD19 mAb (R&D system). The percentages of CD19+/ROR1+ MEC-ROR1 cell was determined by 4-color, multiparameter flow cytometry, which were in turn analyzed by FlowJo software (TreeStar). Finally, the total number of MEC-ROR1 cells per spleen was calculated by multiplying the percentage of MEC-ROR1 in the spleen of each mice and total spleen cell count.

## RESULTS

**14-3-3ζ interact with ROR1 receptor in CLL cell.**

In this experiment, we used Mass Spectrum(MS) analysis technique to identify the protein that interact with ROR1. Among the peptides that were digested from anti-ROR1 immune precipitate prepared from freshly isolated CLL cells, one of which yeilded the MS diagram that is consisant with that of 14-3-3ζ (figure 2A). Futhermore, we probed the ROR1 targeting immune precipitate with anti-14-3-3ζ antibody, and the immunoblot result detected the presence of 14-3-3ζ. Vice versa, we also probed the anti-14-3-3ζ immune precipitate with the anti-ROR1 antibody successfully (figure 2B).

**ROR1 receptor interact with 14-3-3ζ through the S857 motif.**

Here, we found that the amino acid sequence of the ROR1 receptor contains two serine rich sequences RSPSSA/RSPSSAS with the serine at the site of 857(S857). More importantly, the S857 motif resembles the 14-3-3 binding motifs of other known 14-3-3 binding proteins (figure 3A). Furthermore, the S857 motifs of the ROR1 receptor is conserve between a variety of vertebrates (figure 3B).

To confirm whether the S857 motif of ROR1 is required for its binding with 14-3-3ζ, we mutated the serine in the S857 motif of ROR1 to alanine (S857A) and transfected the wild type/mutated ROR1 into MEC1 cell line. While the anti-ROR1 immune precipitate prepared from the WT MEC1-ROR1 cell lysate was probed successfully with anti-14-3-3ζ antibody, no protein was found when we use antibody targeting 14-3-3ζ to probe the anti-ROR1 immune precipitate prepared from the cell lysate of MEC1-ROR1 S857A (figure

3C). According to the confocal result, the mutation of S857 motif of ROR1 receptor disrupted the colocalization of 14-3-3ζ with ROR1 that was originally observed in WT MEC1-ROR1 cell (figure 3D).

**The recruitment of 14-3-3ζ onto ROR1 receptor is induced by Wnt5a.**

We isolated the CLL cell from patients' body freshly and visualized the relative position of ROR1 and 14-3-3 using confocal microscopy. Here, we detected the merging between ROR1 and 14-3-3ζ in CLL cell treated with control Immunoglobulin G (Ctrl-IgG). However, treating the CLL cell with Cirmtuzumab (UC-961), a humanized monoclonal antibody that targets ROR1 receptor, eliminated the merging between ROR1 with 14-3-3ζ (figure 4A/B).

After starving the CLL cell of the patient sample overnight, we used confocal assay again to measure the localization of ROR1 and 14-3-3ζ in the CLL cells that receive the combination treatments of Ctrl-IgG, UC-961, and Wnt5a. According to the confocal results, the ROR1 and 14-3-3ζ receptor in CLL receiving Ctrl IgG treatment no longer colocalized together after the overnight starvation. The Wnt5a treatment, on the other hand, reintroduced the colocalization in between. Furthermore, the combination treatment of Wnt5a with UC-961 on CLL cell disrupted the merging between ROR1 and 14-3-3ζ (figure 4C/D).

**14-3-3ζ mediates Wnt5a induced recruitment of ARHGEF2 onto ROR1 in CLL.**

We probed the ARHGEF2 targeting immune precipitate with anti-14-3-3ζ antibody, and the immunoblot result detected the presence of 14-3-3ζ protein. Vice versa, we

probed the anti-14-3-3ζ immune precipitate with the anti-ARHGEF2 antibody, and the immunoblot pattern also showed the existence of ARHGEF2 (figure 5A).

In the present of Wnt5a, we visualize the merging between ROR1 and ARHGEF2 in overnight starved CLL cell using confocal assay. The colocalization of ROR1 and ARHGEF2 was found as the CLL was transfected with control siRNA. When we introduce siRSNA specified for 14-3-3ζ into the Wnt5a treated CLL cell, the merging between 14-3-3ζ and ROR1 was lost (figure 5B).

**14-3-3ζ is required for the Wnt5a induced RhoA/Rac1 activation by ARHGEF2 in CLL.**

After we treated the overnight starved CLL cell with Wnt5a, we found that the immune precipitate generated from the resulting cell lysis probed with anti-ARHGEF2 antibody showed increased exchange activity for both RhoA and Rac1 (figure 6A). Later, we treated starved CLL cell transfected with siRNA specific for 14-3-3ζ with Wnt5a and measured the RhoA/Rac1 exchange of the resulting anti-ARHGEF2 immune precipitate. Consequently, the Wnt5a induced enhancement of RhoA/Rac1 exchange of immune precipitate prepared from the 14-3-3ζ siRNA treated CLL is significantly reduced (figure 6A).

We also monitor the global activation of RhoA/Rac1 by probing the anti-GTP-RhoA/Rac1 immune precipitate generated from the cell lysis of overnight staved CLL with the antibody specify for GTP-RhoA/Rac1. According to the result of the pull down assay, the starved CLL cell treat with Wnt5a showed enhanced amount of RhoA/Rac1

exchanged with GTP. However, the CLL transfected with 14-3-3ζ siRNA show less degree of GTP-RhoA/Rac1 increment after the Wnt5a treatment (figure 6B).

**14-3-3ζ is required for the noncanonical Wnt signaling mediated migration and proliferation of MEC1 cell.**

Using transwell assay and CCK-8 kit, we quantified the migration of MEC1 based on the percentage of membrane crossing cell in responds to CCL21 stimulation and the proliferation of MEC1 according to the cell number obtained from OD value of cell culture. Compared with the wild type MEC1 cell, the MEC cell transfected with ROR1 showed stronger proliferation and migration. When we introduce the 14-3-3ζ targeting CRISPER-CAS9 construct into both types of cell, the migration/proliferation advantage for MEC-ROR1 cell in compare to MEC1 cell was greatly weakened (figure 7A/B).

We further investigate the global RhoA/Rac1 activation in MEC1 cell using the immune precipitate assay. Similar to the result of proliferation and migration, MEC1-ROR1 had higher level of RhoA/Rac1 activation than MEC1. While the MEC-ROR1-Δ14-3-3ζ also had more activated RhoA/Rac1 than MEC1-Δ14-3-3ζ does, the difference is not as great as the one between the MEC1 and MEC-ROR1.

## DISCUSSION

In this study, we found that 14-3-3ζ is used to recruit ARHGEF2 on to the ROR1 receptor in CLL cell. The 14-3-3ζ expression level is positively correlated with the aggressiveness of the CLL disease because its high expression was found in the CLL patients with poor prognostic outcome (UM IGHV) and fast disease progression (short TTX). While the 14-3-3ζ can be pull down with ROR together using the cell lysis prepared from CLL, the mutation of S857, the potential 14-3-3ζ binding site on ROR1, prevented both the pull down and the colocalization between ROR1 and 14-3-3ζ in MEC-ROR1 cell. Furthermore, the treatment of anti-ROR1 antibody UC-961 disrupted the merging of ROR1 with 14-3-3ζ observed in freshly isolated CLL cell. Although the overnight starvation of the CLL cell eliminated the colocalization between ROR1 and 14-3-3ζ, treating CLL with Wnt5a can retained the colocalization in between. Lastly, the colocalization between the 14-3-3ζ and ROR1 in CLL can be disrupted by the treatment of 14-3-3 siRNA. Based on results being mentioned above, we conclude that 14-3-3ζ paly a potential role in promoting the CLL development. More importantly, we discover that the Wnt5a induced activation of ROR1 receptor facilitates its anchoring with 14-3-3ζ via the S857 motif, which results in the recruitment of ARFGEF2.

### 14-3-3ζ binds physically with ROR1 receptor via its S857 motif in CLL

According to the MS analysis result, we confirm the existence of 14-3-3ζ among the ROR1 bounded proteins using the CLL cell isolated freshly from the patients (figure 2A). Also, 14-3-3ζ was pulled down together with ROR1 from the fresh CLL lysis

successfully using the immunoprecipitation assay (figure 2B). These two observations suggest that 14-3-3ζ can binds physically with ROR1 receptor in CLL. The impact of 14-3-3ζ on CLL disease progression and its binding with ROR1 in CLL collaboratively prompt us to suspect that 14-3-3ζ influences CLL proliferation and chemokine-induced migration by mediating the cellular signaling pathway involving the Wnt induced activation of ROR1 (Yu et al., 2015).

Similar to regular 14-3-3ζ binding ligands which contain the phosphoserine rich 14-3-3ζ binding motif (Muslin, 1996), ROR1 is also identified to have a potential 14-3-3ζ binding motif S857 that is rich in serine (figure 3A). The conservation of S857 motif across the ROR1 receptor of a variety of animal species further indicates its significance in regulating the cellular function of the organism (figure 3B). The mutation of the S857 motif of the ROR1 receptor in MEC cell not only prevented 14-3-3ζ from being pull down with ROR1 but also disrupted the colocalization of ROR1 with 14-3-3ζ observed in MEC1 cell with WT ROR1 receptor (figure 3C/D). Hence, the motif S857 is necessary to mediate the interaction between 14-3-3ζ and ROR1 receptor.

**Wnt induced activation of ROR1 anchors 14-3-3ζ**

In body environment, the ROR1 receptor of CLL cell is continually phosphorylated to be activated. However, the anti-ROR1 antibody can inactivate ROR1 receptor by dephosphorylating ROR1 (Hojjat-Farsangi., 2003). Here we found that treating the freshly isolated CLL cell with UC-961 eliminateed the colocalization of ROR1 with 14-3-3ζ observed in Ctrl-IgG treated fresh CLL cell (figure 4A/B). This result suggests that 14-3-3ζ

binds to ROR1 when the receptor is continually activated. In contrast, the antibody induced inactivation of ROR1 decreases its interaction with 14-3-3ζ. Hence, the activation of ROR1 receptor is necessary to anchor 14-3-3ζ.

Wnt5a treatment of CLL cell retained the colocalization of ROR1 and 14-3-3ζ that was originally lost during the overnight starvation (Figure 4C). This indicates that the binding of ROR1 receptor with 14-3-3ζ disrupted by the starvation induced inactivation of the ROR1 receptor can be reverted by the treatment of Wnt5a. Since Wnt5a binds ROR1 receptor to activate the noncanonical Wnt signaling pathways (Fukuda et al., 2008), Wnt-induced activation of ROR1 receptor is required for the anchoring of 14-3-3ζ onto ROR1.

UC-961 is able to neutralize the enhanced proliferation effect induced by Wnt5a on CLL cells (Yu et al., 2015). Base on that, we propose that UC-961 can act as a competitive antagonist to block the Wnt induced activation of ROR1 receptor and the downstream anchoring of 14-3-3ζ. As what we expect, the application of UC-961 onto the Wnt5a treated CLL cell caused the pre-existed merging of ROR1 with 14-3-3ζ to decrease significantly (figure 4C).

**14-3-3ζ act as an adaptor protein to recruit ARHGEF2 onto Wnt5a activated ROR1 receptor in CLL.**

It has been reported that Wnt5a induced activation of ROR1 receptor recruits ARHGEF2 in CLL (Yu et al., 2015). Since the colocalization between ROR1 and ARHGEF2 in Wnt5a treated CLL was disrupted by the knockdown of 14-3-3ζ using siRNA (figure 5B),

we suspect that the 14-3-3ζ mediate Wnt5a induced recruitment of ARHGEF2 on to ROR1 receptor.

Previous research shows that 14-3-3ζ is able to interact with ARHGEF2 in HEK293 cells (Jin et al., 2004). This interaction also happens in CLL because ARHGEF2 can be pulled down with 14-3-3ζ together using the cell lysis prepared from the freshly isolated CLL cell (figure 5A). Since 14-3-3ζ is anchored on to the ROR1 receptor directly with Wnt5a activation, we propose that 14-3-3ζ help to recruit ARHGEF2 on to ROR1 receptor in CLL using the following mechanism: The Wnt5a induced activation of ROR1 receptor triggers the anchoring of 14-3-3ζ onto ROR1, which in turn causes 14-3-3ζ to interact and recruit ARHGEF2 onto ROR1.

Upon the binding of ROR1 by Wnt5a, not only ARHGEF2 but also ARHGEF1 and ARHGEF6 have been reported to be recruited onto the ROR receptor. However, the knock down of 14-3-3ζ did not disrupt the recruitment of ARHGEF1 and ARHGEF6 onto ROR1 receptor. As a result, the mechanism for the recruitment of other subtypes of GEFs is required for further investigation. Previous research shows that the SH3 domain of ARHGEF6 mediate its interaction with P21 protein-activated kinase 1(PAK1) in HeLa cell (Manser., et al). Since the intracellular PRD of ROR1 receptor contains several SH3-binding sites, ARHGEF6 might be anchored directly on to the PRD of ROR1 via the SH3 domain. Since ARHGEF1 do not have SH3 domains (Rossman., et al), it may require an adaptor protein similar with 14-3-3 to be recruited onto ROR1 receptor.

**The Wnt5a-induced recruitment of ARHGEF2 by 14-3-3ζ onto ROR1 triggers its RhoA/Rac1 exchange activity in CLL**

Previous research shows that the anti-ARHGEF2 immune precipitate made from the Wnt5a treated CLL had increased exchange activity for RhoA /Rac1 (Yu et al., 2015), which suggests that Wnt5a enhances the ability for ARHGEF2 to exchange RhoA/Rac1. In figure 6A, the knock-down of 14-3-3ζ using siRNA inhibited the Wnt5a induced enhancement of RhoA /Rac1 exchange by ARHGEF2. Therefore, we conclude that 14-3-3 is required for Wnt5a induced RhoA/Rac1 activation by ARHGEF2 in CLL. Since the Wnt5a induced recruitment of ARHGEF2 onto the ROR1 receptor is also mediate by 14-3-3ζ, the anchoring of ARHGEF2 by 14-3-3ζ during the recruitment step mediates its enzymatic activity of GTP exchange.

When monitoring global activation of RhoA/Rac1 in CLL using the immune precipitate assay, we make the unexpected observation that the knock-down of 14-3-3ζ only partially reduced the Wnt5a elicited enhancement of RhoA/Rac1 activation (figure 6B). Following the activation by Wnt5a, the ROR1 receptor recruits ARHGEF1 to exchange RhoA and ARHGEF6 to exchange Rac1 (Yu et al., 2015) without using 14-3-3 as the adaptor protein (supplemental figure). Based on these evidences, we conclude that the remaining RhoA/Rac1 activation after the 14-3-3ζ knock-down are results of Wnt5a induced recruitments of ARHGEF1 and ARHGEF6.

**RhoA/Rac1 exchange results in the migration and proliferation of MEC1 cell**

As a B lymphocytic leukemia cell line, the MEC1 is generated artificially to express Wnt5a (Yu et al., 2015). Since the transfection of ROR1 enhances the proliferation and migration of MEC1 cell (figure 7A/B), we speculate that the binding of ROR1 by Wnt5a triggers the noncanonical Wnt signaling pathway to promote the cell proliferation/migration. The knock-out of 14-3-3ζ by CRISPER-CAS9 construct, on the other hand, lessens the ROR1 transfection induced MEC1 proliferation/migration elevation (figure 7A/B). Thus, 14-3-3ζ plays a potential role in the Wnt5a induced migration and proliferation of MEC1 cell.

Previous research shows that the activation of RhoA and Rac1 contributes separately to the enhanced chemokine-induced leukemia migration and cell proliferation (Cuesta-Mateos et al, 2010) (Liu et al, 2006). Since RhoA/Rac1 activation in respond to Wnt5a was controlled by 14-3-3ζ mediated recruitment of ARHGEF2 onto ROR1, we derive that 14-3-3ζ mediates the migration/proliferation of MEC1 in the following way: upon Wnt5a activation, the ARHGEF2 recruited by 14-3-3ζ onto ROR1 caused the enhancement of migration and proliferation through the RhoA/Rac1 activation.

FIGURES

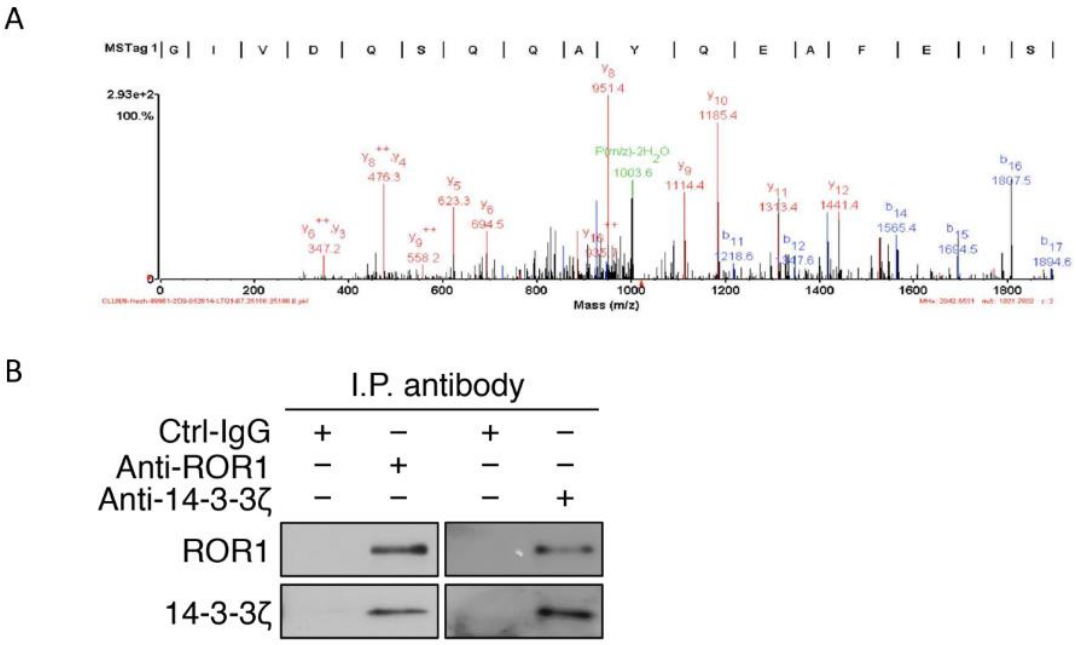


**Figure 1. ROR1 bind with 14-3-3ζ in CLL cell**

A). the Mass Spectrum of 14-3-3ζ that is detected from the ROR targeting immune precipitates prepared from the lysates prepared from the freshly isolated CLL. B). ROR1 Immunoblot of the anti-ROR1(left)/anti 14-3-3ζ(right) immune precipitate in CLL cell is shown in the top row; 14-3-3ζ immunoblot of the anti-ROR1(left)/anti 14-3-3ζ(right) immune precipitate is shown in the bottom row. All the immune precipitates were prepared from the cell lysate of the freshly isolated CLL.

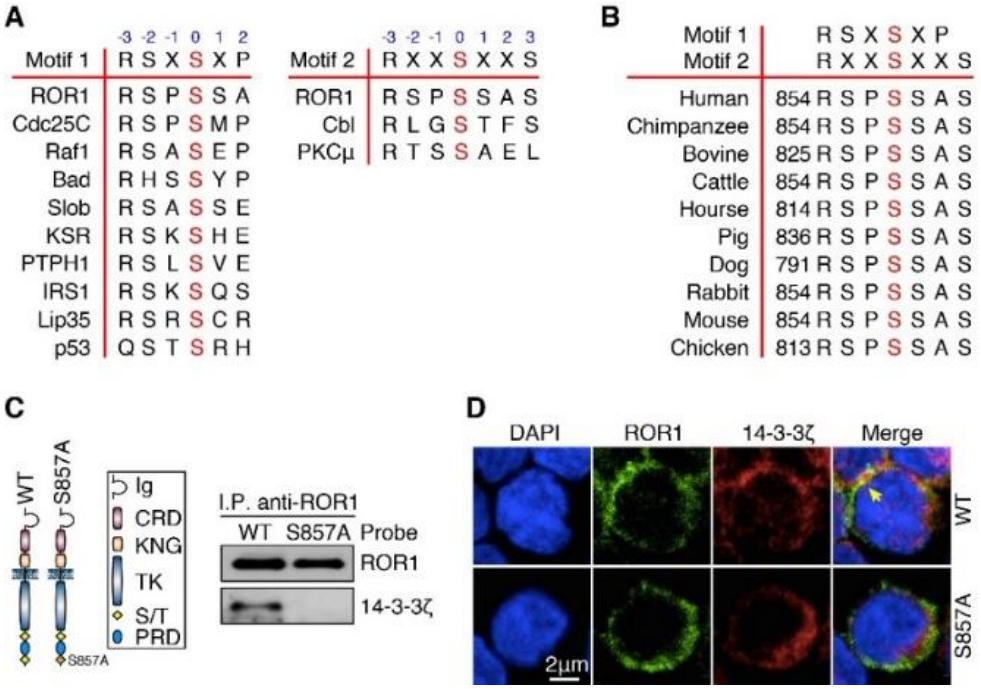


**Figure 2. the S857 motif of ROR1 mediate its interaction with 14-3-3ζ**

A). the proteins that contains the motif S857. B). the animal species that have S857 motif in their ROR1 receptors. C). schematic depicts the structure of wild type ROR1 (WT) and the ROR1 with the S857 mutation(S857A). The anti-ROR1 immune precipitates prepared from the lysates of MEC1-ROR1(left) and MEC1-ROR1 S857A (right) are probed with ROR1(top) and 14-3-3ζ (bottom). D). Colocalization of 14-3-3ζ (green) with WT ROR1/ROR1 S857A (red) detected by confocal microscopy in MEC1-ROR1 cell line.

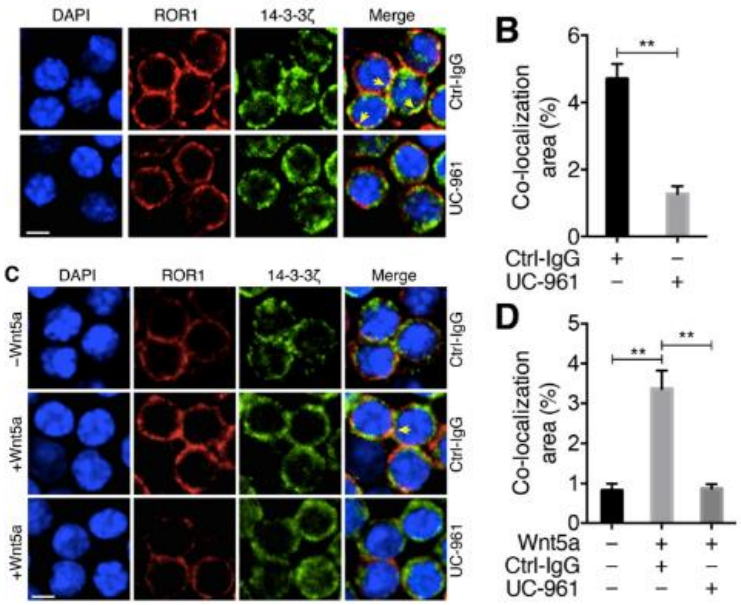


**Figure 3. the Wnt induced activation of ROR1 recruit 14-3-3ζ**

A). colocalization of 14-3-3ζ (green) with ROR1 (red) detected by confocal microscopy in freshly isolated CLL cell with the treatment of either Ctrl-IgG (top) or UC-961 (bottom). B). the average percentage of colocalizations area for 14-3-3ζ with ROR1 in fresh CLL cells treated with Ctrl-IgG (black bar) or UC-961 (gray bar) are calculated as the proportion of merging fluorescence compared to the total fluorescence. C). overnight starved CLL cell was treated with Ctrl-IgG or UC-961 with (+) or without (–) Wnt5a in order to visualize the colocalization of 14-3-3ζ with ROR1 under the confocal microscopy. D). the average percentage of colocalizations area for 14-3-3ζ with ROR1 in overnight starved CLL cells that are treated with Ctrl-IgG (black bar), Wnt5a/Ctrl-IgG (gray bar) and Wnt5a/UC-961 (brown bar). Data in figure B and D are shown as mean ± SEM. *P < 0.05

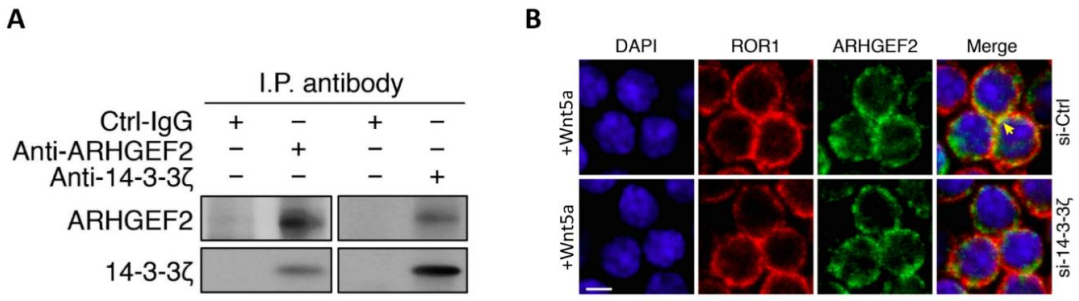


**Figure 4. the knock-out of 14-3-3ζ disrupt the Wnt5a-induced recruitment of ARHGEF2 onto ROR1**

A). ARHGEF2 Immunoblot of the anti-ARHGEF2 (left)/anti 14-3-3ζ(right) immune precipitate in CLL cell is shown in the top row; 14-3-3ζ immunoblot of the anti- ARHGEF2 (left)/anti 14-3-3ζ(right) immune precipitate is shown in the bottom row. All the immune precipitates were prepared from the cell lysate of the freshly isolated CLL. B). the confocal microscopy is used to detect the colocalization of ROR1 (red) and ARHGEF2 (green) in overnight starved CLL cell treated with or without Wnt5a and si-Ctrl or si-14-3-3ζ.

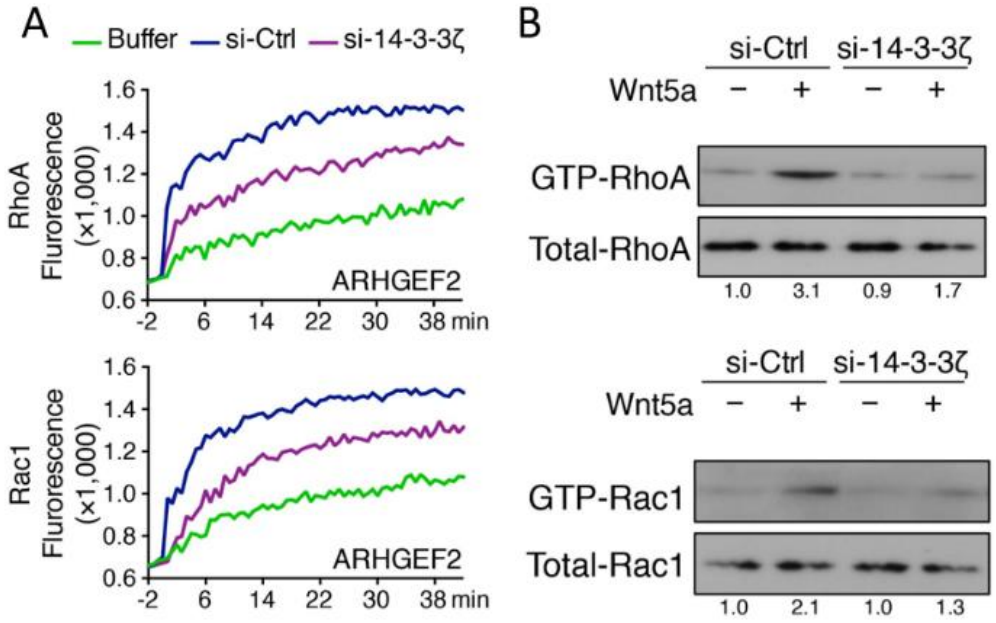


**Figure 5. the knock-out of 14-3-3ζ inhibits the Wnt5a induced activation of RhoA and Rac1 by ARHGEF2 in CLL**

A). The anti-ARHGEF2 immune precipitates prepared from the cell lysates of Wnt5a-treated CLL transfected with si-Ctrl (blue lines) or si-14-3-3ζ (purple lines) were measure for their exchange of RhoA (top) and Rac1 (bottom) over time. The green lines represent the exchange of RhoA/Rac1 using buffer alone. B). RhoA/Rac1 activation by ARHGEF2 in CLL cell without (–) or with (+) Wnt5a treatment transfected with Ctrl-siRNA or siRNA specific for 14-3-3ζ. The total RhoA or Rac1 is determined by running the whole cell lysate in parallel to that of the activated one on the same gel. The ratio of band density for activated versus total RhoA/Rac1 is calculated as the number indicated at the bottom of each lane

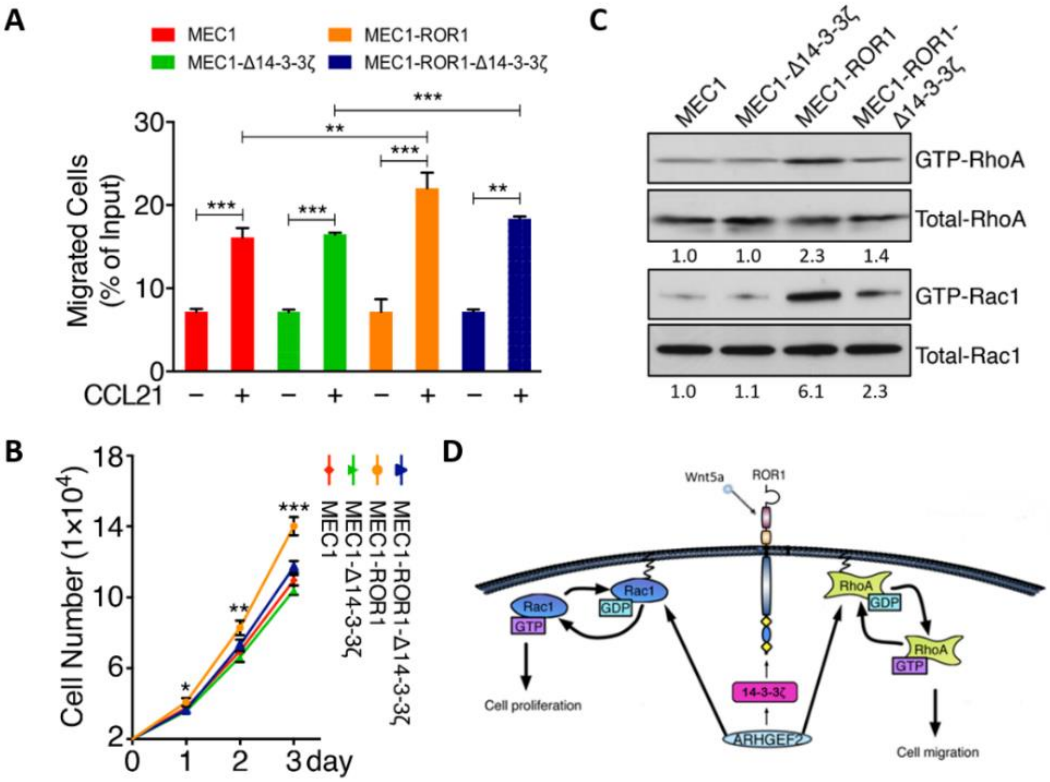


**Figure 6. the knock-out of 14-3-3ζ reduces the ROR1 mediated proliferation and migration of MEC1 in vitro**

A). Bars indicate the mean proportions of MEC1/ MEC1-ROR1 with or without the knock-out of 14-3-3ζ using CRISPER assay (various colors, as indicated in the legend) passing the porous membrane of the transwell assay treated with (+) or without (–) CCL21, as indicated at the bottom. B). Mean numbers of MEC1 /MEC1-ROR1 with or without the 14-3-3ζ knock out (as various symbols indicated in the legend) in pantalets wells at the days indicated at the bottom. Data in panel C and D are shown as mean ± SEM. **P < 0.01; ***P < 0.001, as determined by 2-tailed Student's t test. C) RhoA/Rac1 activation in MEC1/MEC- ROR1 cell with or without the knock-out of 14-3-3ζ. The whole cell lysate was run in parallel for the determination of the total RhoA/Rac1. The number below each lane represents the ratio of the band density for activated versus total RhoA/Rac1 normalized to that of the WT MEC1. D). Proposed model for Wnt5a-induced enhancement of proliferation and migration of MEC1 cell

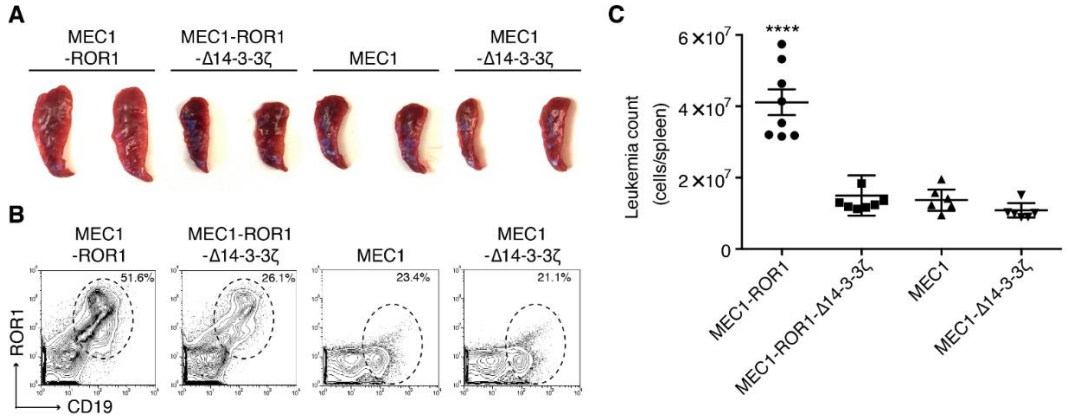


**Figure 7. 14-3-3ζ knock-out reduced the ROR1 mediated engraftment of MEC in vivo**

A). the spleens of Rag2−/−γc−/− mice two weeks after 5 × $10^6$ MEC1/MEC-ROR1 cell with or without 14-3-3ζ knocked out was administrated via i.v infusion. B). the contours show the fluorescence of MEC1/MEC-ROR1 cell with or without 14-3-3ζ knocked out stained with 4A5–Alexa Fluor 647 (ordinate) and anti-CD19-PE (abscissa). All cells were collected from the spleens of mice infused with MEC1/MEC-ROR1 two weeks earlier. The proportion of cells with fluorescence above the threshold indicated by the dotted line is shown as the percentage at the top right corner of each image. C). the counts of CD19+ MEC-ROR1 cells harvested from the spleens of the mouse (n=6) injected with WT MEC-ROR1 (left) or CRISER MEC-ROR1 (right). Data are shown as mean ± SD. **P < 0.01; ***P < 0.001, as determined by 2-tailed Student's t test.

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